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PREDICTION AND TESTING OF ANTIGENIC SITES OF THE AIDS VIRUS
HTLV-III, RECOGNIZED BY T-LYMPHOCYTES FOR THE DEVELOPMENT
OF POSSIBLE SYNTHETIC VACCINE

FINAL REPORT

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## FOREWORD

In conducting research using animals, the investigator(s)
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Whether the immune protective mechanisms against AIDS involve
antibodies or cytotoxic T lymphocytes (CTL) or both, helper T cells will be
necessary to induce either type of response. Also, it is likely that CTL will be
essential to control infection by destroying infected monocytes and
macrophages which provide a reservoir of virus and from which virus can
spread from cell to cell without ever being freely extracellular where it would
be accessible to antibody. For these reasons, T-cell immunity of both the
helper and cytotoxic types will be essential goals of any vaccine strategy.
Whole virus and subunits prepared from virus may be too dangerous to use
as vaccine candidates. Much work has focused on recombinant forms of the
envelope protein. However, the whole envelope protein has certain
disadvantages even when made by recombinant DNA technology. It contains
B-cell epitopes that elicit antibodies that are not neutralizing but rather
detrimental in that they can enhance viral uptake by Fc receptor binding to
macrophages and monocytes, and also may contain epitopes that induce
suppressor T cells that inhibit the immune response. Our strategy has been to
identify appropriate helper T-cell epitopes, CTL epitopes, and neutralizing
antibody epitopes and design an artificial construct made by peptide synthesis
or possibly by recombinant DNA methodologies that would contain repeats of
such epitopes (to increase immunogenicity over that induced by the natural
monovalent protein), and possibly copies of homologues from different viral
isolates (to increase the spectrum of HIV viruses for which the vaccine would
be effective), and to exclude deleterious sites inducing suppression or
enhancing antibodies.

As a first step in that endeavor, we have been identifying helper T and
CTL epitopes on the envelope protein, and have begun such work on the
internal proteins of the *tat* and *pol* genes. Our work on model proteins led, in
collaboration with the theoretical biology group of Charles DeLisi, to a
predictive algorithm which searches for regions of protein sequence which, if
folded as a helix, would be amphipathic, that is, would have one side
hydrophobic and one side hydrophilic. We have found that such structures
have a significantly higher than random chance of being immunodominant

T-cell epitopes (DeLisi and Berzofsky, *Proc. Natl. Acad. Sci. USA* 82: 7048, 1985; Margalit *et al., J. Immunol.* 138: 2213, 1987: Spouge *et al., J. Immunol.* 138: 204, 1987). By our latest count, among sites whose localization was no. biased by use of any such algorithm, 34/48 immunodominant helper T-cell epitopes (p < 0.003) and 7/7 CTL epitopes (p < 0.002) identified in the literatur? have this property (Cornette *et al., Methods in Enzymology* 178: 611-634, 1989). We have therefore analyzed the sequence of proteins from HIV and have been synthesizing and testing peptides from these proteins.

Helper T-cell epitopes:

We first identified two helper T-cell epitopes in the HIV envelope that were predicted and came from relatively conserved regions of the envelope. These were called envT1 (residues 428-443) and envT2 (residues 112-124), and were shown to be recognized by proliferating T cells from several strains of mice of different major histocompatibility complex (MHC) types, immunized with a recombinant protein containing most of the sequence of gp120, the external glycoprotein of the envelope (R10, prepared by Scott Putney, Repligen) (Cease *et al., Proc. Natl. Acad. Sci. USA* 84: 4249, 1987). One of these, envT1 was also shown to be able to immunize mice of three different MHC types to induce T cells that proliferate in response to the native gp120 protein purified from virus (prepared by Gerry Robey, Larry Arthur, Peter Nara, Steve Pyle, and Peter Fischinger of the NCI-FCRF facility). This ability to immunize with a synthetic peptide and elicit T-cell immunity against the native viral protein is essential for a vaccine.

We have now tested these peptides in several other systems in primates. First, we have shown that the majority of 12 chimpanzees immunized with either native gp120 or a recombinant fragment PB1 (corresponding to residues 294-474, made by Scott Putney, Repligen) respond to either envT1 or envT2 or both by proliferation of peripheral blood T lymphocytes *in vitro*, and have shown that these peptides also induce IL-2 secretion (K. Krohn *et al.*, manuscript in preparation). Of the 12 animals (8 immunized with gp120 and 4 with PB1), 8 responded to envT1 and 11 responded to a slightly longer peptide, 426-450, containing the envT1 site, and 9 responded to envT2.

Second, we have shown that immunization of African green and Rhesus monkeys (in collaboration with Phil Markham of Bionetics and Ron Desrosiers, New Eng. Regional Primate Center, respectively, and Peter Nara, NCI) with mixtures of these peptides and a third peptide (TH4.1 or HP53) corresponding to residues 834-848 of gp160 (for lack of enough monkeys to test each separately) can induce T-cell help *in vivo* such that when the animals are challenged with the whole protein (gp120 or gp160, respectively) they make an enhanced antibody response compared to monkeys that had not received peptide before whole protein (A. Hosmalin *et al.*, manuscript in

preparation). These results indicate that these epitopes induce T-cell help, not just proliferation, and that they can successfully immunize primates, not just mice. Moreover, we have found that immunization of the monkeys with mixtures of the peptides elicits peripheral blood T cells that respond *in vitro* to the whole gp120 or gp1( protein, again as one would want a vaccine component to do. Also, there seems to be a correlation between the monkeys that demonstrated T-cell proliferation to the peptides and those that manifested help for an antibody response.

Third, we have tested, in a *retrospective* study (approved by the NCI IRB), the peripheral blood T-cell proliferative responses *in vitro* of cells from human volunteers who had been immunized by Daniel Zagury in Paris and Zaire with a recombinant vaccinia virus expressing the gene for the whole gp160 envelope protein, and then boosted with soluble protein (with approval of the French and Zairian governments). Of 14 HLA-diverse outbred donors, 11 had been recently boosted. Eight of these 11 responded to envT1 *in vitro*, and 4 to envT2, compared to none of 3 controls who had not been immunized, and none of the 3 who had not been boosted (Berzofsky *et al.*, *Nature*, 334: 706, 1988). This is a surprisingly large proportion of an outbred MHC-diverse population, and suggests that envT1 will be a useful T-cell epitope in the majority of humans as well as mice, and that envT2 will also be useful. The sample size was too small and the diversity of HLA types too great to see any correlation with HLA type. Furthermore, we found that those who had been boosted with a soluble protein fragment of gp120 bound to a monoclonal antibody (9284, developed by Biotech and now marketed by Dupont) made significantly higher T-cell proliferative responses than those boosted with soluble fragment only. These results suggest a more effective way to induce T-cell immunity for which there is an experimental basis *in vitro* but which has not been tried before in humans (Berzofsky *et al*, *Nature*, 334: 706, 1988).

Fourth, in addition to the two epitopes studied above, we have prepared a large series of 44 synthetic peptides from gp160, both ones predicted on the basis of amphipathicity and ones not predicted, and have tested these for stimulation of lymph node T cells from mice of 4 distinct MHC types immunized with whole recombinant gp160. We have identified six multideterminant regions, including the two above, that contain clusters of immunodominant helper T-cell epitopes, that are seen by either 3/4 or all 4 MHC types of mice (P. Hale *et al.*, *International Immunology* 1: 409, 1989). The regions correspond to residues 109-134, 324-356, 428-451, 483-506, 787-820, and 828-863. Therefore, the widespread concern based on model protein studies, that individual peptide epitopes may not be useful in an MHC-diverse outbred population because only a small fraction of the population would respond to any given peptide, may be unfounded. Epitopes that are immunogenic in a large number of MHC types would be extremely valuable in a vaccine. Four of the six regions are in relatively conserved segments of

the envelope protein, and five of the six (all except 483-506) were predicted on the basis of amphipathicity.

Fifth, we have finally been able to study the peripheral blood T-cell responses of HIV-infected patients. This had been difficult previously because most infected individuals, even 80% of those still in Walter Reed stage I, with normal T cell numbers and no symptoms, have already lost the ability of their T-cells to proliferate in response to soluble protein antigens, such as tetanus toxoid or influenza antigens. Now, in collaboration with Gene Shearer's lab, however, we have found that as many as 50% of Walter Reed stage one HIV-seropositive people retain T-cell responses to these soluble antigens as measured by the production of the lymphokine, interleukin-2 (IL-2) (Clerici et al., *Nature* 339: 383, 1989). This discovery is of basic and practical interest in its own right, because it suggests a difference in the signal requirements for activation of T cells for IL-2 production compared to proliferation, or the possibility of an inhibitor of proliferation in HIV infection that does not affect IL-2 production. The finding may also be of prognostic value, since patients who retain both functions may be at an earlier stage or have a better prognosis than ones who have lost one of these functions; to determine the prognostic value will require longitudinal follow-up studies of these individuals over time. In addition, this finding finally allowed us to study the 50% of patients who can respond by IL-2 production to control antigens like flu or tetanus toxoid to see if they develop helper T-cell immunity to synthetic peptide T-cell epitopes of the HIV-1 envelope during natural infection. Four of these peptides were studied with cells from 21 unrelated, HLA-diverse HIV-seropositive asymptomatic blood donors who retained such IL-2 production to control antigens. We found that 67% responded to the T1 site, 57% to T2, 60% to TH4.1, and 50% to the P18 site (Clerici et al. *Nature* 339: 383, 1989). These are large fractions, considering that this is an outbred HLA-diverse population. Moreover, 85-90% of the donors responded to at least one of these 4 peptides. Thus, to cover an such a diverse outbred population such as humans, it may not be necessary to incorporate in a synthetic vaccine large numbers of T-cell epitopes; rather, a small number such as 4 to 6 may be sufficient. This is consistent with the findings above in mice that some regions of the envelope can be seen by T cells from individuals of multiple MHC types.

Sixth, in a collaborative study with Drs. Tom Palker and Bart Haynes at Duke, we coupled the T1 helper T-cell site to the major neutralizing antibody epitope of the HIV-1 envelope, called SP10 (described by Palker and Haynes and others), and showed that the peptide T1-SP10 induced antibody responses in mice to SP10 without an additional carrier much better than SP10 alone. Furthermore, in goats this construct induced much higher titers of neutralizing antibodies than the SP10 peptide coupled to a large protein carrier, tetanus toxoid (Palker et al., *J. Immunol.* 142: 3612, 1989). Thus, the T1 epitope that we discovered not only induces T-cell proliferation and IL-2

production in mice and humans, but also serves as a carrier to induce help for a neutralizing antibody response. This T1-SP10 construct is a prototype of the simplest type of synthetic vaccine that we are trying to design. Obviously, a final vaccine would need additional helper T-cell sites, probably additional antibody epitopes and certainly ones from different viral isolates to account for the variability of the virus, as well as CTL epitopes. The optimal arrangement of these in a synthetic vaccine is yet to be determined, but this simplest construct represents the first step in demonstrating the feasibility of such an approach.

**Cytotoxic T lymphocyte (CTL) epitopes:**

In addition to the helper T-cell epitopes, we have identified the first CTL epitope of any AIDS viral protein (Takahashi *et al., Proc. Natl. Acad. Sci. USA* 85: 3105, 1988), in a collaboration with two other groups at NIH. Using mice immunized with a recombinant vaccinia virus expressing gp160 (made by Bernie Moss, NIAID), and restimulating the immune spleen cells *in vitro* with fibroblasts transfected with the gp160 gene (made by Joe Cohen and Ron Germain, NIAID), we induced CTL that would kill the transfected MHC-identical fibroblasts but not control fibroblasts. H-$2^d$ mice were high responders and surprisingly H-$2^k$ mice were low or nonresponders for CTL to the entire envelope protein when immunized and tested this way. We screened 44 synthetic peptides, using the method of Townsend *et al.* (*Cell* 44: 959, 1986) to sensitize untransfected target cells with synthetic peptides, and found that only one of these 44 sensitized targets for killing by H-$2^d$ envelope-specific CTL, and none did so for cells from immune nonresponder H-$2^k$ mice. CTL specific for this peptide were found to be CD8$^+$CD4$^-$, and to be restricted to only one class I MHC molecule, D$^d$. We have been able to clone long-term T-cell lines specific for this epitope. The use of only one of the 5 class I MHC molecules available between the two MHC types, and the recognition of only one immunodominant epitope in association with this molecule, suggests that the CTL repertoire for the HIV envelope may be much more limited than the helper T-cell repertoire. This one immunodominant CTL epitope corresponds to residues 315-329 of the HIV envelope (Ratner numbering) (and is called peptide 18 or simply P18). It is unfortunately in a highly variable region of the sequence. Interestingly, though, this corresponds almost exactly to the site identified independently by three groups as the major neutralizing antibody site on the envelope. Thus, the same peptide may be useful to elicit both CTL and neutralizing antibodies, although variant peptides from many different HIV isolates may be necessary in a vaccine because of the variability of this region.

This immunodominant CTL epitope of the HIV-1 envelope that we found in H-$2^d$ mice we have now also found to be recognized by human CD8-positive T-cells from vaccinated individuals (immunized with a recombinant

vaccinia virus expressing gp160) (Achour *et al.*, manuscript in preparation), as well as from the peripheral blood of HIV-infected donors of Walter Reed stages 1 and 2 (Clerici *et al.*, manuscript in preparation). It appears in both groups of individuals that this epitope, corresponding to the peptide P18, is seen in association with very common human class I MHC molecules, such as HLA-A2. Thus, this epitope is also relevant to vaccines for humans. Moreover, every epitope that we first identified in mice and then studied with human T cells was recognized by human T cells. Thus mice represent a good model for identifying sites useful in humans. However, this site is in a hypervariable portion of the protein sequence.

We were therefore interested in studying the effect of this variation on CTL recognition. We made murine CTL clones to two different isolates, HIV-1 IIIB and HIV-1 MN, and found that they did not crossreact with each other or with a third isolate, RF. To examine the molecular basis of this lack of crossreactivity, we synthesized a series of peptides each with the sequence of the epitope of the IIIB isolate, except for a single substitution from the RF isolate. Where the two were identical, we substituted an alanine. Most of these substitutions had little effect, but three of them significantly reduced killing (Takahashi *et al.*, *J. Exp. Med.*, 170: 2023, 1989). This could occur if the substitution affects binding of the peptide to the class I MHC molecule on the target cell, or affects binding to the receptor of the CTL. To distinguish these mechanisms, we used the inactive mutant peptides to compete for presentation of the wild-type IIIB peptide to IIIB-specific CTL. Only one of the three substituted peptides competed, indicating that this can still bind the MHC molecule, whereas the other two substitutions appear to abrogate binding to the MHC molecule (Takahashi *et al.*, *J. Exp. Med.*, 170: 2023, 1989). Interestingly, the latter two residues are conserved between the IIIB and MN isolates of HIV-1, whereas the former substitution, which does not affect MHC binding and therefore probably affects CTL activity by affecting recognition by the T-cell receptor, differs between IIIB and MN. To see the effect of this residue on T-cell specificity, we prepared mutant peptides identical to the IIIB and MN peptides (which differ in 6 of 15 positions) except for this single residue which was interchanged between them. This single residue interchange completely and reciprocally reversed the specificity of the two CTL lines (Takahashi *et al.*, *Science*, 246: 118, 1989). We conclude that much of the T-cell receptor specificity is focused on this single amino acid residue. Further, we have thus demonstrated that HIV naturally mutates both residues that interact with the T-cell receptor and those that interact with the MHC molecule. Either type of mutation would lead to escape from existing CTL. If the patient had already lost T-cell help, then mutation of a residue interacting with the receptor of existing CTL might be sufficient for escape. However, if help were still competent to elicit new CTL, then new CTL could still control the virus. In contrast, a mutation in a residue necessary for interaction of the epitope with the MHC molecule would prevent recognition by any CTL. Since an individual has only a small fixed

number of HLA alleles, and cannot generate a diverse repertoire of HLA molecules as one can for T-cell receptors or antibodies, such a mutation would make that epitope of the virus invisible to the CTL immune system of that individual. CTL have been shown by Letvin and colleagues to suppress viral replication. Because we have found that there are relatively few immunodominant CTL epitopes, and it is known that the virus can mutate in vivo during the course of infection of a single individual, such mutations in just a few key sites may be sufficient for the virus to escape from immune control and cause AIDS. This is a testable hypothesis, and we would like to study CTL isolated at different times during the course of infection, in relation to serial isolates of virus from the same individual.

Interestingly, we have now found that this same peptide, P18, also contains a helper T-cell epitope which is either identical to, or largely overlapping, the CTL epitope. During in vitro restimulation of envelope-vaccinia-immune BALB/c or B10.D2 spleen cells with peptide in the absence of exogenous lymphokine, the peptide 18 induces T-cell help for stimulation of CTL to itself (Takahashi et al., J. Exp. Med., in press, 1990). The help is by CD4-positive T cells and is restricted by the I-A$^d$ class II MHC molecule, whereas the CTL elicited are CD8-positive and restricted by the class I molecule D$^d$. After CD4$^+$ cell depletion or blocking with anti-I-A antibodies, the help can be replaced by addition of recombinant IL-2. The help is under genetic control. Thus, untreated immune spleen cells from the B10.A mouse (which expresses the appropriate class I molecule, D$^d$, but an inappropriate class II molecule, I-A$^k$) behave just like CD4-depleted immune spleen cells from the B10.D2 or BALB/c mouse, which has I-A$^d$ as well as D$^d$. It is interesting to speculate whether this dual function of eliciting help as well as CTL may contribute to the immunodominance of this site in the BALB/c mouse. Thus, P18, from the V3 loop of gp160, appears to be very versatile, being the site of a major neutralizing antibody epitope, an immunodominant CTL epitope in both mice and humans, and a helper T-cell epitope that induces help for itself in mice, and can elicit IL-2 production in humans (see above, and Clerici et al., Nature 339: 383, 1989). We are currently using substituted peptides to try to determine whether the same amino acid residues are responsible for binding to class II MHC molecules and induction of help as are involved in binding to class I MHC molecules and recognition by CTL.

Finally, it is important to identify T-cell epitopes from proteins other than the envelope. The envelope has been the focus of much attention because it is exposed on the surface of infected cells and of virions and is the target of neutralizing antibodies. However, T cells are not limited to seeing cell surface proteins. Indeed, as shown by Townsend (cited above), most of the CTL response to influenza is directed to internal viral proteins not found on the surface of infected cells. Instead, T cells see fragments of proteins that

are produced as degradation products, and associated with cell-surface MHC molecules. In the case of helper T cells, these are usually exogenously taken up proteins processed in endosomes and associated with class II MHC molecules. In the case of CTL, these are usually endogenously synthesized proteins that are degraded in the cytoplasm, exact site unknown, and associated with class I MHC molecules (see Berzofsky, in *The Antigens*, M. Sela, editor, Vol. VII, pp. 1-146, Academic Press, New York, 1987; Germain, *Nature* 322, 687, 1986; and Berzofsky et al., *Immunol. Rev.* 106: 5-31, 1988). The advantage of these internal proteins over the envelope (as pointed out by Gordon Ada at the 4th International Conference on AIDS in Stockholm in June, 1988) is that these can elicit CTL without inducing antibodies that will enhance viral uptake into macrophages. If, as recent data suggest, even neutralizing antibodies will not be protective, another strategy will be to focus on inducing CTL, in the absence of antibodies, that can control infection and prevent disease even if they do not actually prevent infection. Even if one is not so pessimistic about antibodies as Gordon Ada in his talk, it has been clear from the work of Townsend and others that internal proteins are important targets of CTL immunity. Therefore, we have analyzed the sequences of the protein products of the *gag* and *pol* genes of HIV-1, and have begun making peptides that correspond to predicted sites, using the algorithm described above. Ron Germain (NIAID) has also been making transfected fibroblasts expressing these genes, and Bernie Moss's lab (NIAID) has made recombinant vaccinia viruses containing these genes. Therefore, we plan to continue this three-way collaboration to use similar methods to identify CTL epitopes from these internal viral proteins.

In our first progress in studying CTL epitopes of non-envelope proteins, we have just identified a new CTL epitope in the reverse transcriptase protein of HIV-1, using recombinant vaccinia viruses from Bernie Moss and a transfectant made by Ron Germain, in this collaborative effort. With vaccinia viruses containing truncated *pol* gene inserts, and synthetic peptides covering the region, Anne Hosmalin in my lab has identified this new site seen by CD8 cytotoxic T cells in association with class I MHC molecules, and has defined its limits with a series of truncated peptides that she made (Hosmalin et al., *Proc. Natl. Acad. Sci. USA*, in press, 1990). This site (residues 203-219) may be especially useful because the virus cannot mutate the reverse transcriptase molecule as readily as it can the envelope and still preserve function, and it is known that the sequence of this region of reverse transcriptase is relatively conserved even among reverse transcriptase genes from other viruses. This peptide epitope was then studied for its ability to sensitize autologous EBV-transformed B-cell targets from HIV seropositive human donors, in a collaboration with Mario Clerici and Gene Shearer. Indeed, unstimulated PBL effector cells from 5/12 HIV-1 seropositive donors were able to lyse targets incubated with this peptide, whereas none of 5 uninfected control donors had such activity (Hosmalin et al., *Proc. Natl. Acad. Sci. USA*, in press, 1990). The CTL activity was blocked by anti-CD8 but not

anti-CD4. Thus, for the second time in just two tries, a CTL epitope first identified in mice was also found to be seen by CTL from humans. This result indicates that mice should be a useful model system in which to identify epitopes of relevance to humans. This epitope may be of value in vaccine development because of its conserved sequence.

**References:** Listed in text. Reprints and preprints of refereed publications funded in part by these funds from the Army Medical Research and Development Command are attached.

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